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(54) Title: NOVEL MUTEINS OF IFN- β

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10      20
M S Y N L L G F L Q R S S N F Q C Q K L
30      40
L W Q L N G R L E Y C L K D R M N F D I
50      60
P E E I K Q L Q Q F Q K E D A A L T I Y
70      80
E M L Q N I F A I F R Q D S S S T G W N
90      100
E T I V E N L L A N V Y H Q I N H L K T
110     120
F L E E K L E K E D F T R G K L M S S L
130     140
H L K R Y Y G R I L H Y L K A K E Y S H
150     160
C A W T I V R V E I L R N F Y F I N R L
T G Y L R N

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(57) Abstract

A IFN- β mutein in which phe (F), tyr (Y), trp (W) or his (H) is substituted for val (V) at position 101, when numbered in accordance with wild type IFN- β , DNA sequences encoding these IFN- β muteins, recombinant DNA molecules containing those DNA sequences operatively linked to expression control sequences and capable of inducing expression of an IFN- β mutein, hosts transformed with those recombinant DNA molecules, pharmaceutical compositions containing IFN- β muteins and methods of using those compositions to treat viral infections, cancer or tumors or for immunomodulation.

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NOVEL MUTEINS OF IFN- β TECHNICAL FIELD OF THE INVENTION

This invention relates to muteins of
5 interferon-beta ("IFN- β ") in which val (V) at position
101, when numbered in accordance with wild type IFN- β ,
is substituted with phe (F), trp (W), tyr (Y) or his
(H).

BACKGROUND OF THE INVENTION

10 Interferons are single chain polypeptides
secreted by most animal cells in response to a variety
of inducers, including viruses, mitogens and
polynucleotides. Interferons participate in regulation
of cell function, and have antiviral, antiproliferative
15 and immunomodulating properties. Native human
interferons are classified into three major types:
 α -IFN (leukocyte), IFN- β (fibroblast) and γ -IFN
(immune). Native IFN- β is produced primarily by
diploid fibroblast cells and in lesser amounts by
20 lymphoblastoid cells.

IFN- β is a glycoprotein. Its nucleic acid
and amino acid sequences have been determined.
(Houghton et al., "The Complete Amino Acid Sequence of
Human Fibroblast Interferon as Deduced Using Synthetic
25 Oligodeoxyribonucleotide Primers of Reverse

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Transcriptase," Nucleic Acids Research, 8, pp. 2885-94 (1980); T. Taniguchi et al., "The Nucleotide Sequence of Human Fibroblast DNA," Gene, 10, pp. 11-15 (1980)). Recombinant IFN- β has been produced and characterized.

5 IFN- β exhibits various biological and immunological activities. One of IFN- β 's biological activities is its antiviral activity. This antiviral activity can be neutralized by antibodies to IFN- β . See EP-B1-41313. Preparation and purification of
10 antibodies to IFN- β is described in EP-B1-41313 and the references cited therein. IFN- β is also able to bind to cells that express interferon receptors, such as Daudi cells or A549 cells.

As a result of these activities, IFN- β has
15 potential applications in immunotherapy, antitumor and anticancer therapies, and antiviral therapies.

Numerous investigations and clinical trials have been and continue to be conducted into the antitumor and anticancer properties of both wild type
20 and recombinant IFN- β . These include treatment of several malignant diseases such as osteosarcoma, basal cell carcinoma, cervical dysplasia, glioma, acute myeloid leukemia, multiple myeloma and Hodgkin's disease. In addition, IFN- β has been shown to cause
25 local tumor regression when injected into subcutaneous tumoral nodules in melanoma and breast carcinoma-affected patients.

IFN- β (wild-type and recombinant) has been tested clinically in a variety of viral infections,
30 including papilloma viruses, such as genital warts and condylomata of the uterine cervix; viral hepatitis, including acute/chronic hepatitis B and non-A, non-B hepatitis (hepatitis C); herpes genitalis; herpes zoster; herpetic keratitis; herpes simplex; viral

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encephalitis; cytomegalovirus pneumonia; and in the prophylaxis of rhinovirus. Clinical trials using recombinant IFN- β in the treatment of multiple sclerosis have also been conducted and IFN- β is approved for sale
5 in the United States for the treatment of multiple sclerosis.

SUMMARY OF THE INVENTION

10 This invention provides muteins of IFN- β wherein the val (V) at position 101, when numbered in accordance with wild type IFN- β , is substituted with phe (F), tyr (Y), trp (W), or his (H). This invention also provides DNA sequences encoding these IFN- β muteins, recombinant
15 DNA molecules containing those sequences operatively linked to expression control sequences and capable of inducing, in an appropriate host, the expression of the IFN- β muteins, hosts transformed with those recombinant DNA molecules and pharmaceutical compositions containing
20 the IFN- β . These compositions are useful in immunotherapy as well as in anticancer, antitumor and antiviral therapies.

BRIEF DESCRIPTION OF THE DRAWINGS

25 Figure 1 depicts the amino acid sequence of the preferred mutein of this invention IFN- β (phe₁₀₁) (SEQ ID NO: 1).

30 Figure 2 depicts the preferred degenerate DNA sequence encoding IFN- β (phe₁₀₁) and the signal sequence of native IFN- β (SEQ ID NO: 2).

Figure 3 shows an analysis of IFN- β (phe₁₀₁) binding to interferon receptor bearing cells. Panels A and B show receptor binding data for ¹²⁵I-IFN- β (phe₁₀₁) and wild
35 type ¹²⁵I-IFN- β , respectively, to Daudi cells. Panels C and D show receptor binding data for ¹²⁵I-IFN- β (phe₁₀₁) and wild type ¹²⁵I-IFN- β , respectively, to A549 cells.

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Figure 4 shows an analysis of IFN- β (phe₁₀₁) and wild type IFN- β by peptide mapping by endoproteinase Lyse-C.

Figure 5 shows that antibodies to wild type IFN- β neutralize the activity of IFN- β (phe₁₀₁) and wild type IFN- β .

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "wild type IFN- β " means an IFN- β , whether native or recombinant, having the normally occurring amino acid sequence of native human IFN- β , as shown, e.g., in EP-B1-41313, Figure 4.

As used herein, "IFN- β mutein" means a polypeptide wherein the val (V) at position 101, when numbered in accordance with wild type IFN- β , is substituted with phe (F), tyr (Y), trp (W), or his (H), preferably phe (F). Our most preferred IFN- β muteins have an amino acid sequence identical to wild type IFN- β at the other residues. However, the IFN- β muteins of this invention may also be characterized by amino acid insertions, deletions, substitutions and modifications at one or more sites in or at the other residues of the native IFN- β polypeptide chain. In accordance with this invention any such insertions, deletions, substitutions and modifications result in an IFN- β mutein that retains an antiviral activity that can be at least partially neutralized by antibodies to wild type IFN- β .

We prefer conservative modifications and substitutions (i.e., those that have a minimal effect on the secondary or tertiary structure of the mutein). Such conservative substitutions include those described by Dayhoff in the Atlas of Protein Sequence and

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Structure 5 (1978), and by Argos in EMBO J., 8, 779-785 (1989). For example, amino acids belonging to one of the following groups represent conservative changes:

- ala, pro, gly, gln, asn, ser, thr;
- 5 - cys, ser, tyr, thr;
- val, ile, leu, met, ala, phe;
- lys, arg, his; and
- phe, tyr, trp, his.

We also prefer modifications or substitutions
10 that eliminate sites for intermolecular crosslinking or incorrect disulfide bond formation. For example, IFN- β is known to have three cys residues, at wild-type positions 17, 31 and 141. United States patent 4,588,585 refers to an IFN- β mutein in which the cys
15 (C) at position 17 has been substituted with ser (S). This substitution can also be utilized in this invention. For example, this invention contemplates an IFN- β mutein having ser (S) substituted for cys (C) at position 17 and val (V) at position 101 substituted
20 with phe (F), trp (W), tyr (Y), or his (H), preferably phe (F), when numbered in accordance with wild type IFN- β .

By "numbered in accordance with wild type IFN- β " we mean identifying a chosen amino acid with
25 reference to the position at which that amino acid normally occurs in wild type IFN- β . Where insertions or deletions are made to the IFN- β mutein, one of skill in the art will appreciate that the val (V) normally occurring at position 101, when numbered in accordance
30 with wild type IFN- β , may be shifted in position in the mutein. However, the location of the shifted val (V) can be readily determined by inspection and correlation of the flanking amino acids with those flanking val₁₀₁ in wild type IFN- β .

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The IFN- β muteins of the present invention can be produced by any suitable method known in the art. Such methods include constructing a DNA sequence encoding the IFN- β muteins of this invention and
5 expressing those sequences in a suitable transformed host. This method will produce recombinant muteins of this invention. However, the muteins of this invention may also be produced, albeit less preferably, by chemical synthesis or a combination of chemical
10 synthesis and recombinant DNA technology.

In one embodiment of a recombinant method for producing a mutein of this invention, a DNA sequence is constructed by isolating or synthesizing a DNA sequence encoding the wild type IFN- β and then changing the
15 codon for val₁₀₁ to a codon for phe (F), trp (W), tyr (Y) or his (H), preferably phe (F), by site-specific mutagenesis. This technique is well known. See, e.g., Mark et al., "Site-specific Mutagenesis Of The Human Fibroblast Interferon Gene", Proc. Natl. Acad. Sci.
20 USA, 81, pp. 5662-66 (1984); United States patent 4,588,585, incorporated herein by reference.

Another method of constructing a DNA sequence encoding the IFN- β muteins of this invention would be chemical synthesis. For example, a gene which encodes
25 the desired IFN- β mutein may be synthesized by chemical means using an oligonucleotide synthesizer. Such oligonucleotides are designed based on the amino acid sequence of the desired IFN- β mutein, and preferably selecting those codons that are favored in the host
30 cell in which the recombinant mutein will be produced. In this regard, it is well recognized that the genetic code is degenerate -- that an amino acid may be coded for by more than one codon. For example, phe (F) is coded for by two codons, TTC or TTT, tyr (Y) is coded

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for by TAC or TAT and his (H) is coded for by CAC or CAT. Trp (W) is coded for by a single codon, TGG. Accordingly, it will be appreciated that for a given DNA sequence encoding a particular IFN- β mutein, there
5 will be many DNA degenerate sequences that will code for that IFN- β mutein. For example, it will be appreciated that in addition to the preferred DNA sequence shown in Figure 2, there will be many degenerate DNA sequences that code for the IFN- β mutein
10 shown in Figure 1. These degenerate DNA sequences are considered within the scope of this invention.

The DNA sequence encoding the IFN- β mutein of this invention, whether prepared by site directed mutagenesis, synthesis or other methods, may or may not
15 also include DNA sequences that encode a signal sequence. Such signal sequence, if present, should be one recognized by the cell chosen for expression of the IFN- β mutein. It may be prokaryotic, eukaryotic or a combination of the two. It may also be the signal
20 sequence of native IFN- β . The inclusion of a signal sequence depends on whether it is desired to secrete the IFN- β mutein from the recombinant cells in which it is made. If the chosen cells are prokaryotic, it generally is preferred that the DNA sequence not encode
25 a signal sequence. If the chosen cells are eukaryotic, it generally is preferred that a signal sequence be encoded and most preferably that the wild-type IFN- β signal sequence be used.

Standard methods may be applied to synthesize
30 a gene encoding an IFN- β mutein according to this invention. For example, the complete amino acid sequence may be used to construct a back-translated gene. A DNA oligomer containing a nucleotide sequence coding for IFN- β mutein may be synthesized. For

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example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary
5 assembly.

Once assembled (by synthesis, site directed mutagenesis or another method), the DNA sequences encoding an IFN- β mutein of this invention will be inserted into an expression vector and operatively
10 linked to an expression control sequence appropriate for expression of the IFN- β mutein in the desired transformed host. Proper assembly may be confirmed by nucleotide sequencing, restriction mapping, and expression of a biologically active polypeptide in a
15 suitable host. As is well known in the art, in order to obtain high expression levels of a transfected gene in a host, the gene must be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression
20 host.

The choice of expression control sequence and expression vector will depend upon the choice of host. A wide variety of expression host/vector combinations may be employed. Useful expression vectors for
25 eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from E.coli,
30 including col E1, pCR1, pBR322, pMB9 and their derivatives, wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages. Useful
35 expression vectors for yeast cells include the 2 μ

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plasmid and derivatives thereof. Useful vectors for insect cells include pVL 941. We prefer pBG311. Cate et al., "Isolation Of The Bovine And Human Genes For Mullerian Inhibiting Substance And Expression Of The Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986).

In addition, any of a wide variety of expression control sequences may be used in these vectors. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors. Examples of useful expression control sequences include, for example, the early and late promoters of SV40 or adenovirus, the lac system, the trp system, the TAC or TRC system, the major operator and promoter regions of phage lambda, for example PL, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

Any suitable host may be used to produce the IFN- β muteins of this invention, including bacteria, fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. More particularly, these hosts may include well known eukaryotic and prokaryotic hosts, such as strains of E.coli, Pseudomonas, Bacillus, Streptomyces, fungi, yeast, insect cells such as Spodoptera frugiperda (SF9), animal cells such as Chinese hamster ovary (CHO) and mouse cells such as NS/O, African green monkey cells such as COS 1, COS 7, BSC 1, BSC 40, and BMT 10, and

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human cells, as well as plant cells in tissue culture. For animal cell expression, we prefer CHO cells and COS 7 cells in cultures and particularly the CHO-DDUKY- β 1 cell line (infra, pp. 18-19).

5 It should of course be understood that not all vectors and expression control sequences will function equally well to express the DNA sequences described herein. Neither will all hosts function
10 equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it. The vector's copy
15 number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. For example, preferred vectors for use in this invention include those that allow the DNA
20 encoding the IFN- β muteins to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, United States Patent 4,470,461, Kaufman and Sharp,
25 "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) or glutamine synthetase ("GS") amplification (see, e.g., United States patent 5,122,464 and European
30 published application 338,841).

In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility
35 with the actual DNA sequence encoding the IFN- β mutein

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of this invention, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the DNA sequences of this invention, their secretion characteristics, their ability to fold the polypeptides correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the DNA sequences.

Within these parameters, one of skill in the art may select various vector/expression control sequence/host combinations that will express the desired DNA sequences on fermentation or in large scale animal culture, for example, using CHO cells or COS 7 cells.

The IFN- β muteins obtained according to the present invention may be glycosylated or unglycosylated depending on the host organism used to produce the mutein. If bacteria are chosen as the host then the IFN- β mutein produced will be unglycosylated. Eukaryotic cells, on the other hand, will glycosylate the IFN- β muteins, although perhaps not in the same way as native IFN- β is glycosylated.

The IFN- β mutein produced by the transformed host can be purified according to any suitable method. Various methods are known for purifying IFN- β . See, e.g., United States patents 4,289,689, 4,359,389, 4,172,071, 4,551,271, 5,244,655, 4,485,017, 4,257,938 and 4,541,952. We prefer immunoaffinity purification. See, e.g., Okamura et al., "Human Fibroblastoid Interferon: Immunosorbent Column Chromatography And N-Terminal Amino Acid Sequence", Biochem., 19, pp. 3831-35 (1980).

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The biological activity of the IFN- β muteins of this invention can be assayed by any suitable method known in the art. Such assays include antibody neutralization of antiviral activity, induction of
5 protein kinase, oligoadenylate 2,5-A synthetase or phosphodiesterase activities, as described in EP-B1-41313. Such assays also include immunomodulatory assays (see, e.g., United States patent 4,753,795), growth inhibition assays, and measurement of binding to
10 cells that express interferon receptors.

The IFN- β mutein of this invention will be administered at a dose approximately paralleling that employed in therapy with wild type native or recombinant IFN- β . An effective amount of the IFN- β
15 mutein is preferably administered. An "effective amount" means an amount capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that the effective amount of IFN- β
20 mutein will depend, inter alia, upon the disease, the dose, the administration schedule of the IFN- β mutein, whether the IFN- β mutein is administered alone or in conjunction with other therapeutic agents, the serum half-life of the composition, and the general health of
25 the patient.

The IFN- β mutein is preferably administered in a composition including a pharmaceutically acceptable carrier. "Pharmaceutically acceptable carrier" means a carrier that does not cause any
30 untoward effect in patients to whom it is administered. Such pharmaceutically acceptable carriers are well known in the art. We prefer human serum albumin.

The IFN- β muteins of the present invention can be formulated into pharmaceutical compositions by

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well known methods. See, e.g., Remington's Pharmaceutical Sciences by E. W. Martin, hereby incorporated by reference, describes suitable formulations. The pharmaceutical composition of the

5 IFN- β mutein may be formulated in a variety of forms, including liquid, gel, lyophilized, or any other suitable form. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

10 The IFN- β mutein pharmaceutical composition may be administered orally, intravenously, intramuscularly, intraperitoneally, intradermally or subcutaneously or in any other acceptable manner. The preferred mode of administration will depend upon the

15 particular indication being treated and will be apparent to one of skill in the art.

The pharmaceutical composition of the IFN- β mutein may be administered in conjunction with other therapeutic agents. These agents may be incorporated

20 as part of the same pharmaceutical composition or may be administered separately from the IFN- β mutein, either concurrently or in accordance with any other acceptable treatment schedule. In addition, the IFN- β mutein pharmaceutical composition may be used as an

25 adjunct to other therapies.

Accordingly, this invention provides compositions and methods for treating viral infections, cancers or tumors, abnormal cell growth, or for immunomodulation in any suitable animal, preferably a

30 mammal, most preferably human.

Also contemplated is use of the DNA sequences encoding the IFN- β muteins of this invention in gene therapy applications.

Gene therapy applications contemplated

35 include treatment of those diseases in which IFN- β is

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expected to provide an effective therapy due to its antiviral activity, e.g., viral diseases, including hepatitis, and particularly HBV, or other infectious diseases that are responsive to IFN- β or infectious agents sensitive to IFN- β . Similarly, this invention contemplates gene therapy applications for immunomodulation, as well as in the treatment of those diseases in which IFN- β is expected to provide an effective therapy due to its antiproliferative activity, e.g., tumors and cancers, or other conditions characterized by undesired cell proliferation, such as restenosis.

Local delivery of IFN- β using gene therapy may provide the therapeutic agent to the target area while avoiding potential toxicity problems associated with non-specific administration.

Both in vitro and in vivo gene therapy methodologies are contemplated.

Several methods for transferring potentially therapeutic genes to defined cell populations are known. See, e.g., Mulligan, "The Basic Science Of Gene Therapy", Science, 260, pp. 926-31 (1993). These methods include:

- 1) Direct gene transfer. See, e.g., Wolff et al., "Direct Gene transfer Into Mouse Muscle In Vivo", Science, 247, pp. 1465-68 (1990);
- 2) Liposome-mediated DNA transfer. See, e.g., Caplen et al., "Liposome-mediated CFTR Gene Transfer To The Nasal Epithelium Of Patients With Cystic Fibrosis", Nature Med., 3, pp. 39-46 (1995); Crystal, "The Gene As A Drug", Nature Med., 1, pp. 15-17 (1995); Gao and Huang, "A Novel Cationic Liposome Reagent For Efficient Transfection Of Mammalian Cells", Biochem. Biophys. Res. Comm., 179, pp. 280-85 (1991);

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3) Retrovirus-mediated DNA transfer. See, e.g.,
Kay et al., "In Vivo Gene Therapy Of Hemophilia B:
Sustained Partial Correction In Factor IX-Deficient
Dogs", Science, 262, pp. 117-19 (1993); Anderson,
5 "Human Gene Therapy", Science, 256, pp. 808-13 (1992).

4) DNA Virus-mediated DNA transfer. Such DNA
viruses include adenoviruses (preferably Ad-2 or Ad-5
based vectors), herpes viruses (preferably herpes
simplex virus based vectors), and parvoviruses
10 (preferably "defective" or non-autonomous parvovirus
based vectors, more preferably adeno-associated virus
based vectors, most preferably AAV-2 based vectors).
See, e.g., Ali et al., "The Use Of DNA Viruses As
Vectors For Gene Therapy", Gene Therapy, 1, pp. 367-84
15 (1994); United States Patent 4,797,368, incorporated
herein by reference, and United States Patent
5,139,941, incorporated herein by reference.

The choice of a particular vector system for
transferring the gene of interest will depend on a
20 variety of factors. One important factor is the nature
of the target cell population. Although retroviral
vectors have been extensively studied and used in a
number of gene therapy applications, these vectors are
generally unsuited for infecting non-dividing cells.
25 In addition, retroviruses have the potential for
oncogenicity.

Adenoviruses have the advantage that they
have a broad host range, can infect quiescent or
terminally differentiated cells, such as neurons or
30 hepatocytes, and appear essentially non-oncogenic.
See, e.g., Ali et al., supra, p. 367. Adenoviruses do
not appear to integrate into the host genome. Because
they exist extrachromosomally, the risk of insertional
mutagenesis is greatly reduced. Ali et al., supra, p.
35 373.

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Adeno-associated viruses exhibit similar advantages as adenoviral-based vectors. However, AAVs exhibit site-specific integration on human chromosome 19. Ali et al., supra, p. 377.

5 In a preferred embodiment, the IFN- β mutein-encoding DNA of this invention is used in gene therapy for vascular smooth muscle cell proliferation after arterial injury. Injury of the arterial wall results in the migration of smooth muscle cells into
10 the intimal layer of the arterial wall, where they proliferate and synthesize extracellular matrix components. See, e.g., Chang et al., "Cytostatic Gene Therapy For Vascular Proliferative Disorders With A Constitutively Active Form Of The Retinoblastoma Gene
15 Product", Science, 267, p. 518 (1995). This proliferative response has been implicated in the pathogenesis of atherosclerosis.

One clinically significant setting for arterial injury follows percutaneous balloon
20 angioplasty of the coronary arteries. Following mechanical dilation of the artery, in many cases a cellular proliferative response occurs, leading to regrowth of cells locally that impinges on the lumen and compromises blood flow. This response, known as
25 restenosis, has not responded to conventional treatments including antiplatelet agents, angiotensin-converting enzyme antagonists, or cytotoxic drugs in humans. See, e.g., Ohno et al., "Gene Therapy For Vascular Smooth Muscle Cell Proliferation After
30 Arterial Injury", Science, 265, p. 781 (1994).

According to this embodiment, gene therapy with DNA encoding the IFN- β muteins of this invention is provided to a patient in need thereof, concurrent with, or immediately after coronary balloon angioplasty.
35 This approach takes advantage of the antiproliferative

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activity of the IFN- β muteins of this invention to prevent undesired SMC proliferation. The skilled artisan will appreciate that any suitable gene therapy vector containing IFN- β mutein DNA may be used in accordance with this embodiment. The techniques for constructing such a vector are known. See, e.g., Ohno et al., supra, p. 784; Chang et al., supra, p. 522. The coronary balloon angioplasty procedure is well known. Introduction of the IFN- β mutein DNA-containing vector to the target artery site may be accomplished using known techniques, e.g., as described in Ohno et al., supra, p. 784.

In order that this invention may be better understood, the following examples are set forth. These examples are for the purpose of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

Examples

Expression Vector Containing Human IFN- β (phe₁₀₁)

We used plasmid pBG311 as the expression vector. A full description of pBG311 is given in Cate et al., "Isolation Of The Bovine And Human Genes For Mullerian Inhibiting Substance And Expression Of The Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986). The vector uses the SV40 early promoter, splice signal, and polyadenylation signal and was constructed using pAT153 as backbone.

A DNA fragment containing the DNA sequence shown in Figure 2 (SEQ ID NO: 2) was cloned into pBG311 and operatively linked to the SV40 early promoter through a DNA sequence encoding the signal sequence for native IFN- β according to standard protocols. The resulting expression vector was designated pBeta-phe.

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The IFN- β mutein DNA sequence (SEQ ID NO: 2) encodes an IFN- β mutein having an amino acid sequence identical to wild type IFN- β except that the val (V) at position 101, numbered in accordance with wild type IFN- β , is substituted with phe (F). The mutein encoded by this sequence is designated IFN- β (phe₁₀₁).

Competent *Escherichia coli* (SURE[™], Stratagene) were transformed with the pBeta-phe plasmid according to standard procedures. Colonies containing the pBeta-phe plasmid (i.e., containing a DNA sequence encoding IFN- β (phe₁₀₁) were identified by hybridization to a oligonucleotide probe specific for IFN- β (phe₁₀₁) using a standard protocol (Grunstein and Hogness, 1975).

15 Amplification Vector

We used plasmid pAdD26SV(A)-3 to amplify the IFN- β (phe₁₀₁) gene in our ultimate transformants. This plasmid is described in Kaufman and Sharp, "Construction Of A Modular Dihydrofolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982) and in United States patent 4,740,461. The plasmid expresses murine dihydrofolate reductase (DHFR) under the transcriptional control of the Adenovirus 2 (Ad2) major late promoter (MLP). A 5' splice site, derived from an immunoglobulin variable region gene, is located between the Ad2 MLP and the DHFR coding sequences. The SV40 polyadenylation site is present downstream of the DHFR gene. The plasmid contains the prokaryotic origin of replication (ori) and tetracycline resistance gene from pBR322.

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Transformation of a Cell Line

- The CHO-DUKX-B1 DHFR⁻ cell line was cotransformed with the pBeta-phe plasmid and plasmid pAdd26SV(a)-3. This cell line was derived from the
- 5 wild type CHO-K1 cell line by ethyl methanesulfonate and UV irradiation induced mutagenesis. See Chasin and Urlaub, "Isolation Of Chinese Hamster Cell Mutants Deficient In Dihydrofolate Reductase Activity", Proc. Natl. Acad. Sci. USA, 77, pp. 4216-20 (1980).
- 10 Dihydrofolate reductase catalyzes the conversion of folate to tetrahydrofolate. Cells without functional DHFR require exogenous ribonucleosides and deoxyribonucleosides for growth. Inhibition of growth can be induced by methotrexate, a folate analogue,
- 15 which binds to and inhibits DHFR. Titration of methotrexate can lead to methotrexate resistance by amplification of the DHFR gene. (Kaufman & Sharp, 1982, supra): Amplification and increased expression of genes near DHFR often occurs when DHFR is amplified.
- 20 Therefore, cells resistant to high levels of methotrexate often demonstrate increased specific productivity of nearby genes.

- The pBeta-phe plasmid (restricted with Xmn1) and plasmid pAdd26SV(a)-3 (restricted with StuI) were
- 25 mixed in a ratio of 10:1, respectively. The DNA was transformed into CHO-DUKX-B1 DHFR⁻ cells by electroporation. Cells were plated in non-selective α + medium (α MEM base plus ribonucleosides and deoxyribonucleosides, 10% fetal bovine serum [FBS],
- 30 4 mM glutamine) and allowed to grow for 2 days. The medium was then exchanged for α^- medium (α MEM base without ribonucleosides and deoxyribonucleosides, 10% FBS, 4 mM glutamine) + 50 nM methotrexate (MTX). The cells were removed by trypsinization and plated at ca.

- 20 -

8 x 10⁵ cells/10 cm tissue culture plate. After 14 days, clones were picked and grown in 96 well tissue culture plates. One clone was expanded into a 12 well tissue culture plate and then 7 days later put into a 6 well tissue culture plate in the presence of 250 nM MTX. This clone was expanded into a T75 flask (grown in α medium + 250 nM MTX) and then amplified in 750 nM MTX. A subclone was picked into a 96 well tissue culture plate, expanded into a 48 well tissue culture plate, then a 6 well tissue culture plate and then a T75 tissue culture flask.

Purification Of IFN- β (phe₁₀₁)

IFN- β (phe₁₀₁), produced by culturing the above subclone (or others similar to it) and then secreted into the culture medium, can be purified by immunoaffinity chromatography, substantially as described by Okamura et al., "Human Fibroblastoid Interferon: Immunosorbent Column Chromatography And N-Terminal Amino Acid Sequence", Biochem., 19, pp. 3831-35 (1980).

CNBr-Sepharose 4B resin (2 g, 7 ml) is prepared by suspending in 1 mM HCl. The gel is washed with 1 mM HCl for 15 min on a scintered glass filter. Anti-IFN- β mabs (such as B02, Yamasa, Japan) are coupled to CNBr-Sepharose 4B resin by incubating in coupling buffer (100 mM NaHCO₃, pH 8.3, 500 mM NaCl) for 2 hours at room temperature on a rocker platform. Typically, 1-2 mg IFN- β mab per ml of resin is coupled, but this amount can be varied. The unreacted CNBr is blocked with 100 mM Tris-HCl, pH 8, 500 mM NaCl, overnight at 4°C. Alternately, the unreacted CNBr is blocked with 100 mM ethanolamine under substantially the same conditions.

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The coupled resin is washed with three cycles of alternative pH. Each cycle consists of a wash with acetate buffer (100 mM, pH 4) containing 500 mM NaCl followed by a wash with Tris buffer (100 mM, pH 8) containing 500 mM NaCl.

A 1 cm X 3 cm column (2.3 ml bed volume) is prepared with the coupled resin. The column is equilibrated with PBS (greater than 5 column volumes). IFN- β (phe₁₀₁)-containing samples are diluted 1:3 in equilibration buffer, pH 6.8 and loaded. The load is chased with PBS, washed with 20 mM K₂HPO₄, 1 M NaCl, pH 6.8, and eluted with 200 mM Na citrate, pH 2. The pH of the eluate was adjusted to 6 by diluting the sample with 500 mM Mes, pH 6.

15 Characterization By Peptide Mapping

An IFN- β (phe₁₀₁), mutein that had been produced and purified in a different and less preferred manner than described above was characterized by peptide mapping. A 30 μ g aliquot of IFN- β (phe₁₀₁) or wild type IFN- β sample was lyophilized, suspended in 200 μ l of endoproteinase Lys-C digestion buffer (100 mM TRIS, pH 9, 0.5 mM EDTA), incubated for 12 hours at 22° with 1.5 μ g of endoproteinase Lys-C and subjected to mapping analysis on a C₈ reversed phase HPLC column (.45 x 25 cm). The column was developed with a 30 minute, 0-70% gradient of acetonitrile in 0.1% TFA at 1.4 mls/min. The column effluent was monitored at 214 nm. Figure 4, Panel A shows a portion of the peptide map for IFN- β (phe₁₀₁) with the arrowhead indicating the peptide TFLEEK (SEQ ID NO: 3). This peak did not occur in the peptide map for wild type IFN- β . Figure 4, Panel B shows the corresponding region of a peptide map for wild type IFN- β with the arrowhead indicating the

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peptide TVLEEK (SEQ ID NO: 4). The identity of the TFLEEK and TVLEEK were verified by protein sequence analysis. We estimate that the β -Phe₁₀₁ and wild type β -IFN were greater than 98% pure. Protein

5 concentrations were estimated from absorbance at 280 nm using an extinction coefficient of 1.5 for a 1 mg solution. In order to stabilize the proteins for biological studies, they were diluted to 4 μ g/ml in PBS containing 5% FBS and 5 mM HEPES, pH 7.5

10 Antiviral Activity Of IFN- β (phe₁₀₁) In The CPE Assay

The preparation of IFN- β (phe₁₀₁) that was characterized by peptide mapping was analyzed in a Cytopathic Effect (CPE) assay for antiviral activity. A wild type recombinant IFN- β standard was prepared in
15 Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 4mM glutamine at a concentration of 10,000 units/mL and stored in aliquots at -70°C. On day 1, standard, control and IFN- β _{Phe} samples were diluted in DMEM, 10% FBS, 4mM glutamine in three dilution series:
20 i) starting at 64 units/mL followed by 2-fold dilutions, ii) starting at 12 units/mL followed by 1.5-fold dilutions, and iii) starting at 6 units/mL followed by 1.2-fold dilutions. Fifty microliters of the dilutions were then added in columns to the wells
25 of 96-well microtiter plates. A549 cells were added to each well at 10⁵ cells/ml, 50 uL per well, in DMEM, 10% FBS, 4mM glutamine and the cells are incubated at 37°C, 5% CO₂ for 15 to 20 hours.

The plate contents were shaken into a bleach
30 bucket and 100 uL encephalomyocarditis virus (EMC virus) at appropriate dilution in media was added to each well. The virus and cells were incubated at 37°C, 5% CO₂ for 30 hours. The plate contents were then

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shaken into a bleach bucket and 0.75% crystal violet dye added to the plates. After 5 to 10 minutes, the plates were washed with distilled water and allowed to dry before being read visually.

- 5 Samples and standards were tested in duplicate on each assay plate, yielding two data points per dilution series per assay day.

IFN- β (phe₁₀₁) was tested in 14 assays in duplicate. Wild type recombinant IFN- β was used as a
10 standard. Based on these experiments, IFN- β (phe₁₀₁) had a specific activity of 4.8×10^8 U/mg with a 95% confidence interval of $3.5-6.7 \times 10^8$. Wild type IFN- β had a specific activity of approximately 2.0×10^8 units/mg with a confidence interval of $1.6-2.5 \times 10^8$.
15 The data in Figure 5 show a similar result.

The specific activity of recombinant IFN- β (phe₁₀₁) is on average about 2.5 fold higher than that of recombinant wild type IFN- β , as measured in our antiviral assay.

20 Analysis Of IFN- β (phe₁₀₁) For Receptor Binding

The IFN- β (phe₁₀₁) used in the CPE assay above was also analyzed for ability to bind to cells that express interferon receptors. For these studies we examined the binding of either wild type ¹²⁵I-IFN- β or
25 ¹²⁵I-IFN- β (phe₁₀₁) to Daudi cells or A549 cells (Figure 3). Carrier-free IFN- β was iodinated substantially according to the chloramine T method. Unreacted iodine was removed by size exclusion chromatography on a Superdex 75 column that was
30 equilibrated in PBS containing 1 mg/ml bovine serum albumin. The concentration of the iodinated IFN- β was determined by the CPE assay, assuming a specific activity of 2×10^8 units/mg. Normally 5 ng (1 μ L,

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300,000 cpm) of iodinated IFN- β (either alone or in the presence of a 50 fold excess of non-iodinated interferon) was added to 1.7 mL eppendorf tubes in a total volume of less than 10 μ L. The labelled ligand
5 was allowed to bind alone (-) or was competed with unlabeled IFN- β (phe₁₀₁), α 2-IFN (α 2), γ -IFN (γ) or wild type recombinant IFN- β (WT).

Both Daudi cells and A549 cells (American Type Culture Collection) were used. The cells were
10 suspended in DMEM/5% FBS at 2×10^6 cells/mL. To the samples of the IFN- β , 0.5 mL of the cell suspension was added. The tubes were mixed by inversion and incubated at ambient temperature for 45 minutes. The cells were then pelleted at 1000 x g for 2 min and washed two
15 times with 0.5 mL DMEM/10% FBS. Each wash was followed by a centrifugation step at 1000 x g for 2 min. The cells were resuspended in 0.1 mL, transferred into tubes for counting and binding quantified in a Beckman gamma 407 counter.

20 The data suggest that the binding of IFN- β (phe₁₀₁) is very similar to that of wild type IFN- β on both cell types. Comparable amounts of wild type ¹²⁵I-IFN- β and ¹²⁵I-IFN- β (phe₁₀₁) were bound and competed similarly by noniodinated α -IFN, wild type IFN- β and
25 IFN- β (phe₁₀₁). The binding was not affected by the addition of recombinant human γ -IFN.

**The Antiviral Activity Of IFN- β (phe₁₀₁)
Is Substantially Neutralized By Antibodies
To Wild Type IFN- β**

30 A number of preparations of recombinant mutein IFN- β (phe₁₀₁) produced in CHO cells as described above were purified to approximately 90% purity using column chromatography. These samples were diluted to

- 25 -

25 pg/ml. Wild type recombinant IFN- β , designated here as IFN- β (val₁₀₁), was produced and purified in substantially the same way.

Standard antiviral and antibody

5 neutralization assays were used to demonstrate that antibodies to wild type IFN- β (val₁₀₁) at least partially neutralize IFN- β (phe₁₀₁). The particular antiviral assay and neutralization assay that we used, detailed below, are substantially the same as the antiviral and
10 antibody neutralization assays described in European patent EP B1 41313. See p. 26, lines 1-21; p. 29, line 48 - p. 32, line 3.

A. Preparation Of Cell-containing Plates

A549 cells (ATCC CCL185) were seeded into 96
15 well plates at 3×10^4 cells/100 microliter media/well. The media used was Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 4 mM glutamine. The cell containing plates were then incubated at 37°C, 5% CO₂ for approximately 24 hours.

20 B. Preparation Of Master Plates

Master plates containing either sample or standard were then created with serially diluted wells (in duplicate). Sample wells contained either purified recombinant mutein IFN- β (phe₁₀₁) or wild type
25 recombinant IFN- β (val₁₀₁) in the presence or absence of rabbit anti-IFN- β polyclonal sera. Control wells contained either buffer alone or recombinant wild type IFN- β (as a standard) in the presence or absence of an anti-LFA3 antibody (data not shown).

30 Serial dilutions were performed as follows. 200 microliters of control, or of wild type or mutein IFN- β sample (at a concentration of about 25 pg/ml), was added to each well in row A on each plate. The

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final concentration of sample in row A was 8.5 pg/ml.
Dilutions of 1:1.5 were then performed down each plate.

C. Antibodies To Wild Type IFN- β

Antibodies to wild type IFN- β (val₁₀₁) were
5 produced in rabbits immunized with recombinant wild
type IFN- β (val₁₀₁). Rabbit anti-IFN- β polyclonal sera
was collected from the immunized rabbits at appropriate
intervals, pooled and stored until use (Rabbit IFN- β
serum pool 6/25/93; 5 ml/vial, 0.02% azide; ref.
10 0.01742.062).

D. Sample/Ab Incubation

Rabbit anti-IFN- β polyclonal sera was added
to the appropriate wells on the Master plates. The
final dilution of antibody was 1:1000. The
15 antibody/IFN- β mixture was incubated at room
temperature for 45 minutes.

E. Incubation of Cells With Control, Sample Or
Sample/Ab

The media was then aspirated from the
20 cell-containing plates prepared and replaced with
aliquots (100 μ l/well) of control, IFN- β sample or
IFN- β /Ab sample, as appropriate, from the Master plates
prepared. The cell-containing plates were incubated at
37°C, 5% CO₂ for 16-24 hours.

25 F. Viral Challenge

The next step was the viral challenge. The
cell-containing plate contents were then aspirated and
100 μ l of a solution of encephalomyocarditis virus
(EMCV) at appropriate dilution was added to each well.
30 The virus and cells were incubated at 37°C, 5% CO₂ for
41-45 hours.

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The cell-containing plates were developed using the XTT/PMS colorimetric method. A 1 mg/ml XTT (3,3-[1-(phenylamino)carbonyl]-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)-benzenesulfonic acid; Sigma) solution was prepared in phosphate buffered saline solution. A 1 mg/ml PMS (phenazine methosulfate) solution was prepared in water. A PMS/XTT solution was prepared at 1:50. The development solution was prepared by diluting the PMS/XTT 1:3 in phosphate buffered saline solution. The tetrazolium compound XTT is reduced by living cells to form an orange colored formazon. Color formation correlates directly to viable cell number.

The cell-containing plates were aspirated and washed with 150 μ l/well of phosphate buffered saline solution. Each well then received 150 μ l of development solution. The plates were incubated at 37°C, 5% CO₂ for 30-60 minutes.

Absorbance at 450 nm was measured on a Molecular Devices Thermo_{max} microplate reader with Softmax software. The results are displayed graphically in Figure 5. Absorbance is plotted against IFN- β concentration.

Figure 5 shows that in the absence of rabbit anti-IFN- β polyclonal sera, samples of mutein IFN- β (phe₁₀₁) (closed square; ■) and of wild type IFN- β (val₁₀₁) (closed diamond; ♦) protected the A549 cells from the EMCV. This is indicated by the increase in absorbance (indicating more living cells) with increasing mutein IFN- β or wild type IFN- β concentration.

Figure 5 also shows that in the presence of rabbit anti-IFN- β polyclonal sera, samples of mutein IFN- β (phe₁₀₁) (open square; □) and of wild type IFN- β (val₁₀₁) (open diamond; ◇) failed to protect the

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A549 cells from the EMCV. This is shown by the baseline absorbance value for any mutein IFN- β or wild type IFN- β concentration, indicating that almost all A549 cells were dead.

- 5 In sum, Figure 5 shows that the antiviral activity of mutein IFN- β (phe₁₀₁) was neutralized by antibodies to wild-type IFN- β (i.e., rabbit anti-IFN- β antibodies).

Treatment of Restenosis With IFN- β Mutein Gene Therapy

- 10 Initial testing of gene therapy for restenosis is conducted in a pig model using recombinant wild type porcine IFN- β according to the following protocol.

- 15 Cell proliferation is measured by immunohistochemistry. All animals receive an intravenous infusion of BrdC (Sigma, St. Louis, MO), 25 mg/kg total dose, 1 hour before death. Immunohistochemistry with monoclonal antibody to BrdC (1:1000 dilution, Amersham Life Sciences, Arlington Heights, IL) is performed to label nuclei in
20 proliferating cells as described in Goncharoff et al., J. Immunol. Methods, 93, p. 97 (1988). Identification of vascular smooth muscle cells is performed by immunohistochemistry with an antibody to smooth muscle
25 α -actin (1:500 dilution, Boehringer Mannheim, Germany) as described in Isik et al., Am. J. Pathol., 141, p. 1139 (1992).

- Domestic Yorkshire pigs (12 to 15 kg) are anesthetized with zolazepam-tiletamine (6.0 mg/kg) in
30 combination with (2.2 mg/kg intramuscular) rompun with 1% nitrous oxide. The iliofemoral arteries are exposed by sterile surgical procedures, and a double-balloon catheter is inserted into the iliofemoral artery as described in Nabel et al., Science, 249, p. 1285

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(1990). The proximal balloon is inflated to 300 mm Hg, as measured by an on-line pressure transducer, for 5 min. The balloon is deflated and the catheter is advanced so that the central space between the proximal and distal balloon now occupies the region of previous balloon injury. Both balloons are inflated, and the segment is irrigated with heparinized saline. The adenoviral inoculum is instilled for 20 min in the central space of the catheter. The catheter is removed and antegrade blood flow as restored.

The injured arteries of all pigs are infected with 10^{10} plaque-forming units (PFU) per milliliter of an ADV- Δ E1 vector containing an insert encoding porcine IFN- β or with an ADV- Δ E1 vector lacking the insert. In each animal, both iliofemoral arteries are transfected with the same vector at a titer of 1×10^{10} PFU/ml and 0.7 ml is used in each animal (final dose of 7×10^9 PFU).

The vessel segments in these pigs are excised 21 or 42 days later. Each artery is processed in an identical manner. The region of instillation between the two double balloons is cut into five cross-sections of identical size. Sections 1 and 4 are fixed in methyl Carnoy and sections 3 and 5 are fixed in formalin, and all sections are paraffin-embedded and stained with hematoxylin-eosin. Additional antibody studies are performed on methyl Carnoy- or formalin-fixed arteries. Tissue from section 2 is flash-frozen in liquid nitrogen and stored at -80°C for DNA isolation. Measurements of intimal and medial area are determined in four sections from each artery in a blinded manner by two independent readers, and the measurements for each artery are averaged. Slides of arterial specimens are studied with a microscope-based video imaging analysis system (Image-1 System,

- 30 -

Universal Imaging, Weschester, PA) as described in Nabel et al., Proc. Natl. Acad. Sci U.S.A., 90, p. 10759 (1993).

As an alternative to the above-described
5 adenoviral-based Ad- Δ E1 vector, direct gene transfer may also be used. One suitable construct is a plasmid derived from the RSV backbone, with the RSV-LTR promoter driving expression of IFN- β , with the SV-40 poly A' signal 3' to the IFN- β DNA sequence. See,
10 e.g., Gorman et al., Science, 221, pp. 551-53 (1983).

The above protocol is then modified so that the vector contains a DNA insert encoding the human IFN- β muteins of this invention for gene therapy in humans.

15 Sequences

The following is a summary of the sequences set forth in the Sequence Listing:

SEQ ID NO:1 -- Amino acid sequence of IFN- β (phe₁₀₁)
20 SEQ ID NO:2 -- DNA sequence encoding IFN- β (phe₁₀₁), including DNA sequence encoding the signal sequence of native IFN- β
SEQ ID NO:3 -- Amino acid sequence of peptide TFLEEK.
SEQ ID NO:4 -- Amino acid sequence of peptide TVLEEK.

Deposits

25 E.coli K-12 containing plasmid pBeta-phe (which contains a DNA sequence encoding IFN- β (phe₁₀₁) and the native IFN- β signal sequence) has been deposited. The deposit was made in accordance with the Budapest Treaty and was deposited at the American Type
30 Culture Collection, Rockville, Maryland, U.S.A. on

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SEQ ID NO:3 -- Amino acid sequence of peptide TFLEEK.

SEQ ID NO:4 -- Amino acid sequence of peptide TVLEEK.

Deposits

E.coli K-12 containing plasmid pBeta-phe
5 (which contains a DNA sequence encoding IFN- β (phe₁₀₁)
and the native IFN- β signal sequence) has been
deposited. The deposit was made in accordance with the
Budapest Treaty and was deposited at the American Type
Culture Collection, Rockville, Maryland, U.S.A. on
10 March 11, 1994. The deposit received the accession
number 69584.

The foregoing description has been presented
only for purposes of illustration and description.
This description is not intended to limit the invention
15 to the precise form disclosed. It is intended that the
scope of the invention be defined by the claims
appended hereto.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Biogen, Inc.
Goelz, Susan E
Cate, Richard L
Pepinsky, Blake R
Chow, Pingchang E
- (ii) TITLE OF INVENTION: Novel Miteins Of IFN-Beta
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: James F. Haley, Jr.
 - (B) STREET: Fish & Neave, 1251 Avenue of the Americas
 - (C) CITY: New York
 - (D) STATE: New York
 - (E) COUNTRY: USA
 - (F) ZIP: 10020-1104
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Haley Jr., James F
 - (B) REGISTRATION NUMBER: 27,794
 - (C) REFERENCE/DOCKET NUMBER: B179
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (212) 596-9000
 - (B) TELEFAX: (212) 596-9090

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 166 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln
 1             5             10             15

Cys Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cys Leu
      20             25             30

Lys Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys Gln Leu Gln
      35             40             45

Gln Phe Gln Lys Glu Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln
      50             55             60

Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn
      65             70             75             80

Glu Thr Ile Val Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ile Asn
      85             90             95

His Leu Lys Thr Phe Leu Glu Glu Lys Leu Glu Lys Glu Asp Phe Thr
      100            105            110

Arg Gly Lys Leu Met Ser Ser Leu His Leu Lys Arg Tyr Tyr Gly Arg
      115            120            125

Ile Leu His Tyr Leu Lys Ala Lys Glu Tyr Ser His Cys Ala Trp Thr
      130            135            140

Ile Val Arg Val Glu Ile Leu Arg Asn Phe Tyr Phe Ile Asn Arg Leu
      145            150            155            160

Thr Gly Tyr Leu Arg Asn
      165

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(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 561 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION: 1..63

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 64..561

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..561

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATG ACC AAC AAG TGT CTC CTC CAA ATT GCT CTC CTG TTG TGC TTC TOC	48
Met Thr Asn Lys Cys Leu Leu Gln Ile Ala Leu Leu Leu Cys Phe Ser	
-21 -20 -15 -10	
ACT ACA GCT CTT TOC ATG AGC TAC AAC TTG CTT GGA TTC CTA CAA AGA	96
Thr Thr Ala Leu Ser Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg	
-5 1 5 10	
AGC AGC AAT TTT CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG	144
Ser Ser Asn Phe Gln Cys Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg	
15 20 25	
CIT GAA TAC TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC OCT GAG GAG	192
Leu Glu Tyr Cys Leu Lys Asp Arg Met Asn Phe Asp Ile Pro Glu Glu	
30 35 40	
ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC GGC GCA TTG ACC ATC	240
Ile Lys Gln Leu Gln Gln Phe Gln Lys Glu Asp Ala Ala Leu Thr Ile	
45 50 55	
TAT GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT	288
Tyr Glu Met Leu Gln Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser	
60 65 70 75	
AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC	336
Ser Thr Gly Trp Asn Glu Thr Ile Val Glu Asn Leu Leu Ala Asn Val	
80 85 90	
TAT CAT CAG ATA AAC CAT CTG AAG ACA TTC CTG GAA GAA AAA CTG GAG	384
Tyr His Gln Ile Asn His Leu Lys Thr Phe Leu Glu Glu Lys Leu Glu	
95 100 105	
AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC CTG AAA	432
Lys Glu Asp Phe Thr Arg Gly Lys Leu Met Ser Ser Leu His Leu Lys	
110 115 120	

- 35 -

ACA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC AAG GAG TAC AGT	480
Arg Tyr Tyr Gly Arg Ile Leu His Tyr Leu Lys Ala Lys Glu Tyr Ser	
125 130 135	
CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG AAC TTT TAC	528
His Cys Ala Trp Thr Ile Val Arg Val Glu Ile Leu Arg Asn Phe Tyr	
140 145 150 155	
TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA AAC	561
Phe Ile Asn Arg Leu Thr Gly Tyr Leu Arg Asn	
160 165	

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 187 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Thr Asn Lys Cys Leu Leu Gln Ile Ala Leu Leu Leu Cys Phe Ser	
-21 -20 -15 -10	
Thr Thr Ala Leu Ser Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg	
-5 1 5 10	
Ser Ser Asn Phe Gln Cys Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg	
15 20 25	
Leu Glu Tyr Cys Leu Lys Asp Arg Met Asn Phe Asp Ile Pro Glu Glu	
30 35 40	
Ile Lys Gln Leu Gln Gln Phe Gln Lys Glu Asp Ala Ala Leu Thr Ile	
45 50 55	
Tyr Glu Met Leu Gln Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser	
60 65 70 75	
Ser Thr Gly Trp Asn Glu Thr Ile Val Glu Asn Leu Leu Ala Asn Val	
80 85 90	
Tyr His Gln Ile Asn His Leu Lys Thr Phe Leu Glu Glu Lys Leu Glu	
95 100 105	
Lys Glu Asp Phe Thr Arg Gly Lys Leu Met Ser Ser Leu His Leu Lys	
110 115 120	

- 36 -

Arg Tyr Tyr Gly Arg Ile Leu His Tyr Leu Lys Ala Lys Glu Tyr Ser
 125 130 135

His Cys Ala Trp Thr Ile Val Arg Val Glu Ile Leu Arg Asn Phe Tyr
 140 145 150 155

Phe Ile Asn Arg Leu Thr Gly Tyr Leu Arg Asn
 160 165

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Thr Phe Leu Glu Glu Lys
 1 5

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Thr Val Leu Glu Glu Lys
 1 5

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Applicant's or agent's file reference number	B179 CIP PCT	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>31</u> , line S <u>4-11</u> <u>E. coli K-12, JA221/pBeta-phe</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 11 March 1994 (11.03.94)	Accession Number 69584
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>	
In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by requester (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States) EPO	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only <input checked="" type="checkbox"/> This sheet was received with the international application Authorized officer DORIS L. BROOK INTERNATIONAL DIVISION	For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on: Authorized officer
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Applicant's or agent's file reference number	B179 CIP PCT	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>31</u> , line S <u>4-11</u> <u>E. coli K-12, JA221/pBeta-phe</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 11 March 1994 (11.03.94)	Accession Number 69584
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of the designation of Finland, until the application has been laid open to public inspection by the Finnish Patent Office, or has been finally decided upon by the Finnish Patent Office without having been laid open to public inspection, samples of the deposited microorganisms will be made available only to an expert in the art.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
Finland	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer DORIS L. BROOK <i>DLB</i> INTERNATIONAL DIVISION	Authorized officer

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WHAT IS CLAIMED IS:

1. An IFN- β mutein wherein the val (V) at position 101 in wild type IFN- β , numbered in accordance with wild type IFN- β , is substituted with phe (F), tyr (Y), trp (W), or his (H), said mutein displaying an antiviral activity that is at least partially neutralized by antibodies to wild type IFN- β .

2. An IFN- β mutein having an amino acid sequence identical to wild type IFN- β except that the val (V) at position 101 in wild type IFN- β , numbered in accordance with wild type IFN- β , is substituted with phe (F), tyr (Y), trp (W) or his (H).

3. The IFN- β mutein according to claim 2, wherein the val (V) is substituted with phe (F).

4. The IFN- β mutein according to claim 2 having the formula:

Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser-Asn-Phe-Gln-Cys-Gln-Lys-Leu-Leu-Trp-Gln-Leu-Asn-Gly-Arg-Leu-Glu-Tyr-Cys-Leu-Lys-Asp-Arg-Met-Asn-Phe-Asp-Ile-Pro-Glu-Glu-Ile-Lys-Gln-Leu-Gln-Gln-Phe-Gln-Lys-Glu-Asp-Ala-Ala-Leu-Thr-Ile-Tyr-Glu-Met-Leu-Gln-Asn-Ile-Phe-Ala-Ile-Phe-Arg-Gln-Asp-Ser-Ser-Ser-Thr-Gly-Trp-Asn-Glu-Thr-Ile-Val-Glu-Asn-Leu-Leu-Ala-Asn-Val-Tyr-His-Gln-Ile-Asn-His-Leu-Lys-Thr-Phe-Leu-Glu-Glu-Lys-Leu-Glu-Lys-Glu-Asp-Phe-Thr-Arg-Gly-Lys-Leu-Met-Ser-Ser-Leu-His-Leu-Lys-Arg-Tyr-Tyr-Gly-Arg-Ile-Leu-His-Tyr-Leu-Lys-Ala-Lys-Glu-Tyr-Ser-His-Cys-Ala-Trp-Thr-Ile-Val-Arg-Val-Glu-Ile-Leu-Arg-Asn-Phe-Tyr-Phe-Ile-Asn-Arg-Leu-Thr-Gly-Tyr-Leu-Arg-Asn (SEQ ID NO:1).

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5. A DNA sequence encoding an IFN- β mutein where in the val (V) at position 101 in wild type IFN- β numbered in accordance with wild type IFN- β , is substituted with phe (F), tyr (Y), trp (W), or his (H), said mutein displaying an antiviral activity that is at least partially neutralized by antibodies to wild type IFN- β .

6. A DNA sequence encoding an IFN- β mutein having an amino acid sequence identical to wild type IFN- β except that the val (V) at position 101 in wild type IFN- β , numbered in accordance with wild type IFN- β , is substituted with phe (F), tyr (Y), trp (W) or his (H).

7. The DNA sequence according to claim 6, wherein the val (V) is substituted with phe (F).

8. The DNA sequence according to claim 7 encoding an IFN- β mutein of the formula:
Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser-Asn-Phe-Gln-Cys-Gln-Lys-Leu-Leu-Trp-Gln-Leu-Asn-Gly-Arg-Leu-Glu-Tyr-Cys-Leu-Lys-Asp-Arg-Met-Asn-Phe-Asp-Ile-Pro-Glu-Glu-Ile-Lys-Gln-Leu-Gln-Gln-Phe-Gln-Lys-Glu-Asp-Ala-Ala-Leu-Thr-Ile-Tyr-Glu-Met-Leu-Gln-Asn-Ile-Phe-Ala-Ile-Phe-Arg-Gln-Asp-Ser-Ser-Ser-Thr-Gly-Trp-Asn-Glu-Thr-Ile-Val-Glu-Asn-Leu-Leu-Ala-Asn-Val-Tyr-His-Gln-Ile-Asn-His-Leu-Lys-Thr-Phe-Leu-Glu-Glu-Lys-Leu-Glu-Lys-Glu-Asp-Phe-Thr-Arg-Gly-Lys-Leu-Met-Ser-Ser-Leu-His-Leu-Lys-Arg-Tyr-Tyr-Gly-Arg-Ile-Leu-His-Tyr-Leu-Lys-Ala-Lys-Glu-Tyr-Ser-His-Cys-Ala-Trp-Thr-Ile-Val-Arg-Val-Glu-Ile-Leu-Arg-Asn-Phe-Tyr-Phe-Ile-Asn-Arg-Leu-Thr-Gly-Tyr-Leu-Arg-Asn (SEQ ID NO:1).

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9. The DNA sequence according to claim 8 wherein the codon encoding the amino acid at position 101 is TTC.

10. A DNA having the formula of SEQ ID NO:2.

11. A recombinant DNA molecule characterized by the DNA sequence of any of claims 5 to 10, the sequence being operatively linked to an expression control sequence in the recombinant DNA molecule.

12. A host transformed with a recombinant DNA molecule of claim 11.

13. A method of producing an IFN- β mutein wherein the val (V) at position 101 in wild type IFN- β , numbered in accordance with wild type IFN- β , is substituted with Phe (F), tyr (Y), trp (W), or His (H), said mutein displaying an antiviral activity that is at least partially neutralized by antibodies to wild type IFN- β , the method comprising the steps of culturing a host according to claim 12 and collecting the IFN- β mutein.

14. The method according to Claim 13, wherein the IFN- β mutein is encoded by a DNA sequence comprised by the formula of Sequence Id No:2 and the host is an animal cell in culture.

15. A pharmaceutical composition comprising an antiviral, anticancer, antitumor or immunomodulation effective amount of the IFN- β mutein of any one of claims 1 to 4 and a pharmaceutically acceptable carrier.

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M	S	Y	N	L	L	G	F	L	Q	R	S	S	N	F	Q	C	Q	K	L	10	20
L	W	Q	L	N	G	R	L	E	Y	C	L	K	D	R	M	N	F	D	I	30	40
P	E	E	I	K	Q	L	Q	Q	F	Q	K	E	D	A	A	L	T	I	Y	50	60
E	M	L	Q	N	I	F	A	I	F	R	Q	D	S	S	S	T	G	W	N	70	80
E	T	I	V	E	N	L	L	A	N	V	Y	H	Q	I	N	H	L	K	T	90	100
F	L	E	E	K	L	E	K	E	D	F	T	R	G	K	L	M	S	S	L	110	120
H	L	K	R	Y	Y	G	R	I	L	H	Y	L	K	A	K	E	Y	S	H	130	140
C	A	W	T	I	V	R	V	E	I	L	R	N	F	Y	F	I	N	R	L	150	160
T	G	Y	L	R	N																

FIG. 1

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-20 -15 -10 -5
ATGACCAACAAGTGTCTCCTCCAAATTGCTCTCCTGTTGTGCTTCTCCACTACAGCT
M T N K C L L Q I A L L L C F S T T A

1 5 10 15
CTTTCATGAGCTACAACCTTGCTTGGATTCCCTACAAAGAAGCAGCAATTTTCAGTGT
L S M S Y N L L G F L Q R S S N F Q C

20 25 30 35
CAGAAGCTCCTGTGGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGGACAGGATG
Q K L L W Q L N G R L E Y C L K D R M

40 45 50 55
AACTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGACGCC
N F D I P E E I K Q L Q Q F Q K E D A

60 65 70
GCATTGACCATCTATGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAAGATTCA
A L T I Y E M L Q N I F A I F R Q D S

75 80 85 90
TCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAATGTCTATCAT
S S T G W N E T I V E N L L A N V Y H

95 100 105 110
CAGATAAACCATCTGAAGACATTCCTGGAAGAAAACTGGAGAAAGAAGATTTCACC
Q I N H L K T F L E E K L E K E D F T

115 120 125 130
AGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCAT
R G K L M S S L H L K R Y Y G R I L H

135 140 145 150
TACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAATC
Y L K A K E Y S H C A W T I V R V E I

155 160 165
CTAAGGAACTTTTACTTCATTAACAGACTTACAGGTTACCTCCGAAAC
L R N F Y F I N R L T G Y L R N

FIG. 2

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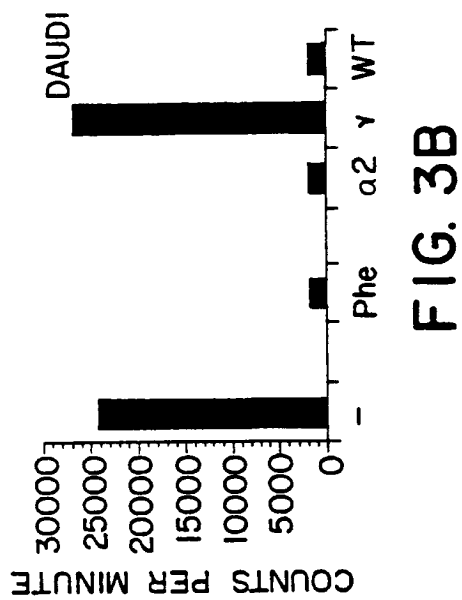


FIG. 3B

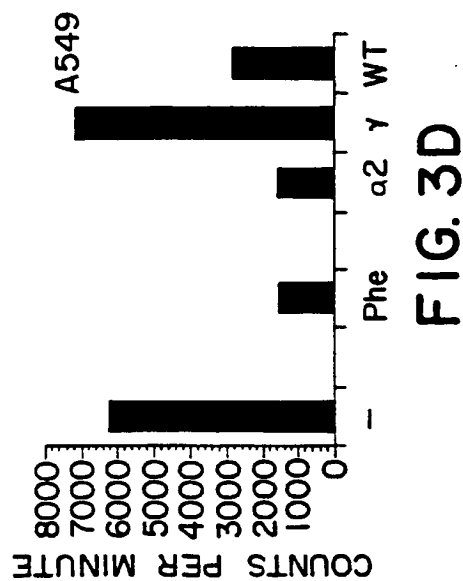


FIG. 3D

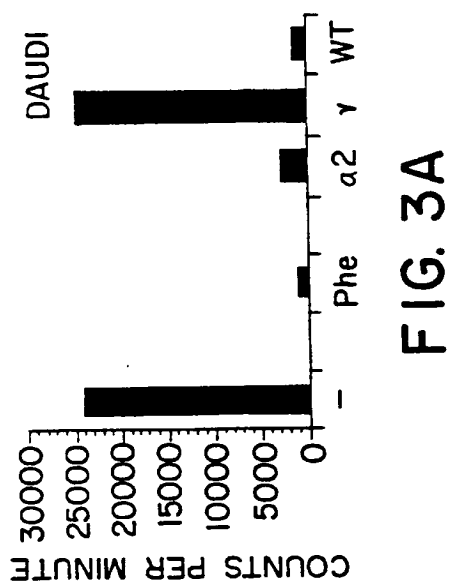


FIG. 3A

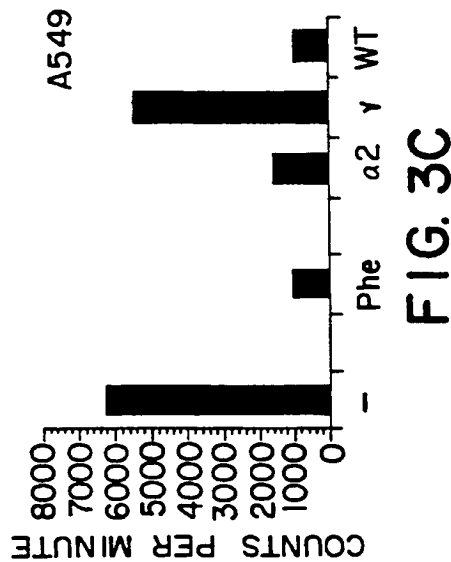


FIG. 3C

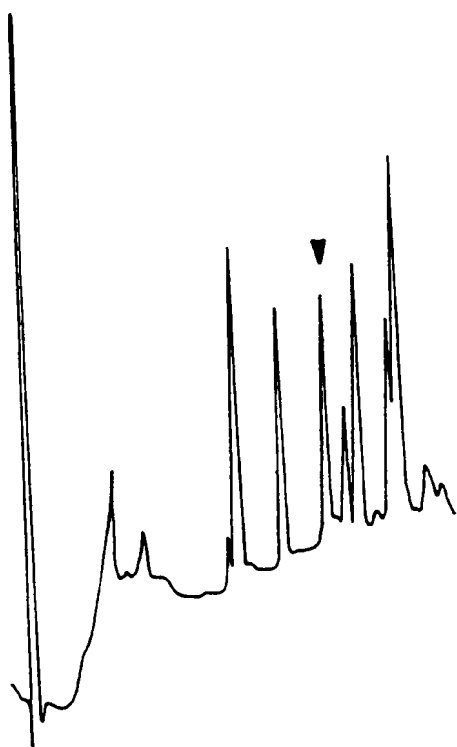


FIG. 4A

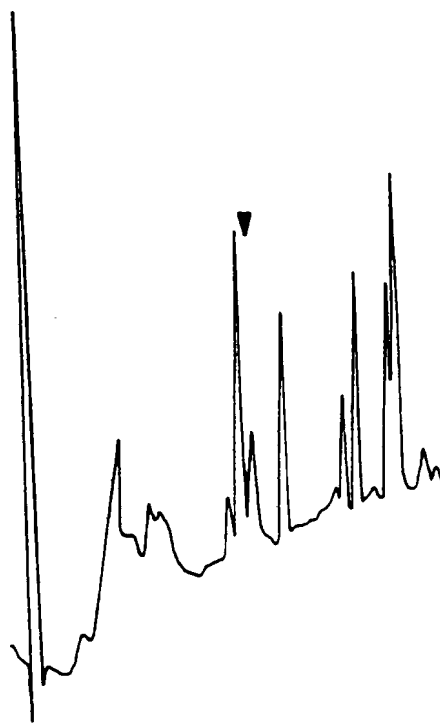


FIG. 4B

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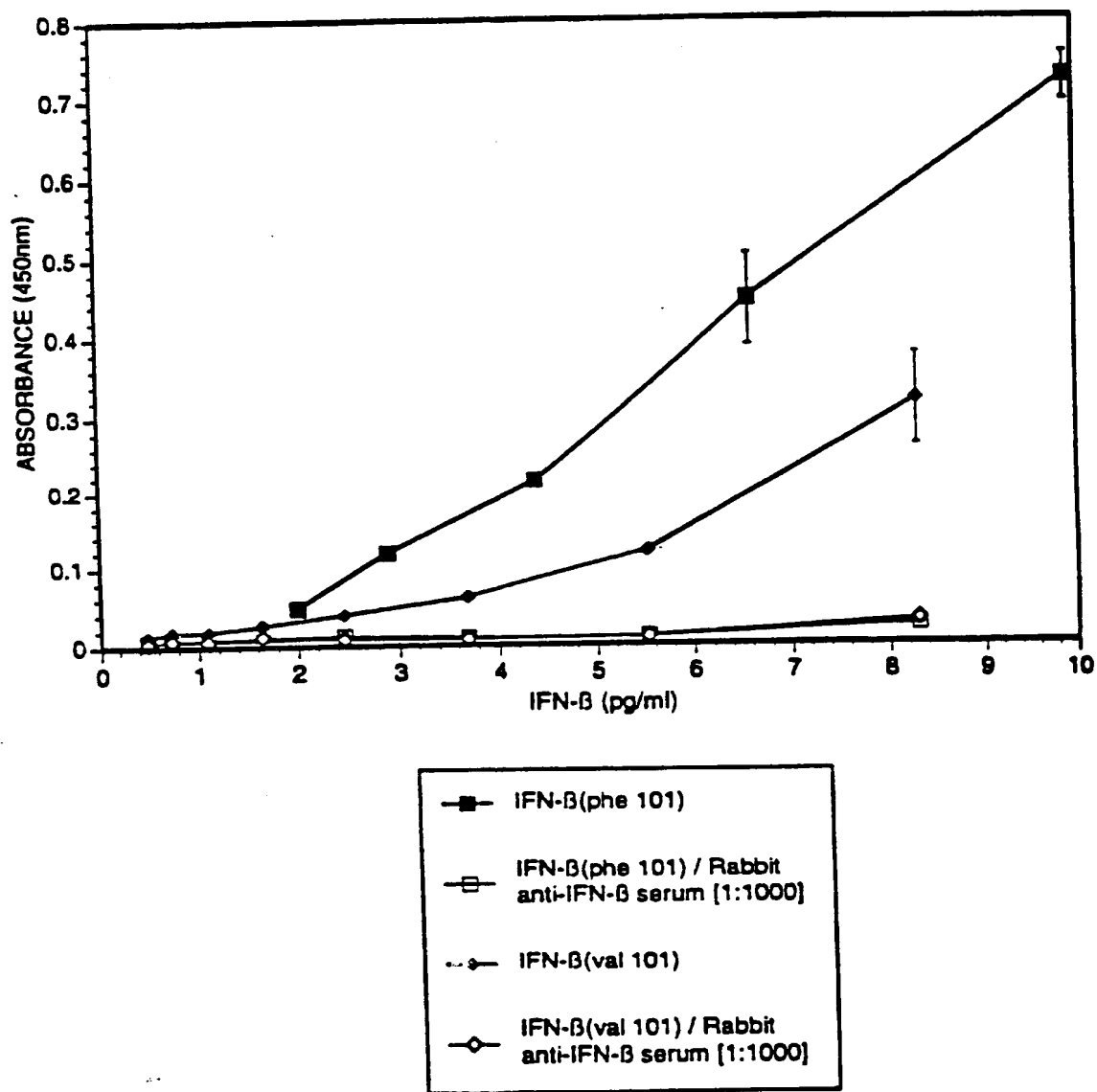


FIG. 5

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 95/03206A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/22 C07K14/565 C12N5/10 A61K38/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 041 313 (BIOGEN N.V.) 9 December 1981 cited in the application see claims see page 82, line 1 - page 83, line 5 see page 89 - page 92, line 30 ---	1-15
A	WO,A,93 15609 (THOMAS JEFFERSON UNIVERSITY) 19 August 1993 --- -/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
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- *O* document referring to an oral disclosure, use, exhibition or other means
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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

20 July 1995

Date of mailing of the international search report

09.08.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Fax (+31-70) 340-3016

Authorized officer

Le Cornec, N

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03206

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.81, no.18, September 1984, WASHINGTON US pages 5662 - 5666 D.F. MARK ET AL 'Site-specific mutagenesis of the human fibroblast interferon gene' cited in the application see the whole document</p> <p>---</p>	1-14
A	<p>CHEMICAL ABSTRACTS, vol. 109, no. 23, 5 December 1988, Columbus, Ohio, US; abstract no. 209388w, WU, SHUHUA ET AL 'A study on the relationship between the N terminal variation of human interferon beta and its antiviral activity' page 515 ;column R ; see abstract & BINGDU XUEBAO, vol.4, no.2, 1988 pages 102 - 106</p> <p>-----</p>	1-15

INTERNATIONAL SEARCH REPORT

Internat'l Application No
PCT/US 95/03206

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0041313	09-12-81	AU-B- 564690	20-08-87
		AU-A- 6897981	08-10-81
		BG-B- 60441	31-03-95
		JP-A- 56154499	30-11-81
		AU-A- 6897881	10-12-81
		BG-B- 60443	31-03-95
		CA-A- 1207251	08-07-86
		DE-A- 3177298	08-04-93
		EP-A, B 0041767	16-12-81
		JP-A- 57014599	25-01-82

WO-A-9315609	19-08-93	NONE	

